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Locust cellular defense against infections: Sites of pathogen clearance and hemocyte proliferation

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ABSTRACT

The locust cellular defense is mediated by hemocytes and hematopoietic tissue. In *Locusta migratoria*, the hemocytes and hematopoietic tissue mutually assist each other in clearing invading pathogens from circulation. A β-1, 3-glucan infection induces nodule formation and apoptotic, TUNEL positive, cells in the hematopoietic tissue and massive loss of hemocytes in the circulation, calling for instant proliferation of hemocytes and hematopoietic tissue cells to assure continued host cellular defense. As the locust hematopoietic tissue persists at the adult stage, it was originally designated as being the major source for the replenishment process. Revisiting post infection hemocyte proliferation, using immunofluorescence based tests for DNA synthesis and mitosis, evidenced the lack of β-1, 3-glucan induced cell proliferation in the hematopoietic tissue. Instead these tests identified the circulating hemocytes as the major source for hemocyte replenishment in the circulation. The hematopoietic tissue, however, undergoes a continuous, slow and infection independent regeneration, thereby accumulating potential phagocytes despite infection, and might serve a prophylactic role in containing pathogens in this swarming insect.

1. Introduction

Besides being an Orthopteran insect model, locusts have huge economic significance as major crop pests in many agrarian communities around the globe (Enserink, 2004; Wang et al., 2014). The locusts, which are normally shy and harmless as solitary animal, can turn into voracious feeders during crowding and can pose a great damage to agriculture (Lovejoy et al., 2006; Pener and Simpson, 2009; Sword et al., 2010). Loss of agricultural crops owing to gregarious locust swarms is a common scenario in many tropical regions of the world, a record of a recent devastating locust outbreak being in Madagascar in 2013 (Monard and Mangione, 2014). Current locust control methods include mainly use of chemical pesticides despite their enormous side-effects on human and the environment (Enserink, 2004). Possible alternative and sustainable solutions such as entomopathogen based biocontrol methods are not still well exploited because of the slow response rate of locusts to pathogen exposure (Lomer et al., 2001; van der Valk, 2007; Wang et al., 2013; Wilson et al., 2002). As the locust immune defense system represents a major player in suppressing the effectiveness of entomopathogens, understanding the locust immunity seems a pivotal step toward the development of an effective locust biocontrol method.

All insects, including locusts, exclusively depend on innate immunity, either cellular or humoral, to defend themselves against invading pathogens (Lavine and Strand, 2002; Tsakas and Marmaras, 2010). The humoral factors such as anti-microbial peptides (AMPs) and some enzyme cascades for coagulation and melanization are produced by fat body or hemocytes (Bulet et al., 1999; Ling and Yu, 2005; Theopold et al., 2002). Such humoral compounds are either freely available in the hemolymph or are released to the hemocoel upon infection to deactivate the pathogen. Apart from this, the insects have macrophage-like hemocytes which can eliminate microbial pathogens via phagocytosis (Jiravanichpaisal et al., 2006; Lavine and Strand, 2002). A large mass of such pathogens can also be contained by the collective action of the hemocytes, in which case the hemocytes form nodules, to restrict the spread of the infection. The insect hemocytes can neutralize large parasitoid infections in a similar fashion by forming capsules around the parasite. Such hemocyte mediated immunities – phagocytosis, nodulation and encapsulation – are considered as insect cellular defense (Lavine and Strand, 2002). The synergistic action of the cellular and humoral responses ensures the complete elimination of

Abbreviation: HPT, Hematopoietic tissue.

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the pathogen (Bulet et al., 1999; Jiravanichpaisal et al., 2006; Lavine and Strand, 2002; Ling and Yu, 2005; Theopold et al., 2002).

In locusts, some humoral factors such as those belonging to the melanization cascades are activated following infection (Goldsworthy et al., 2002; Ratcliffe et al., 1991; Wang et al., 2007, 2013). On the other hand, studies indicated that consecutive or induced AMPs are lacking in the locust hemolymph, although some nine putative AMPs are identified in the recently published genome of *Locusta migratoria* (Wang et al., 2013, 2014). This absence of hemolymph AMPs is linked to a resource allocation during flight stage when the locusts tend to develop a prophylactic immune system. They boost up general scavenging molecules such as pathogen recognition receptors prior to an infection and avoid producing specific effectors such as the AMPs following the infection (Wang et al., 2013). Accordingly, upon infection, the recognized pathogens may be contained by the cellular machinery, which in locusts requires hematopoietic tissue (HPT) and circulating hemocytes (Brehelin and Hoffmann, 1980; Brookman et al., 1989; Hoffmann et al., 1974; Lackie et al., 1985).

Given the lack of some humoral factors, the locusts seem to heavily utilize the cellular defense against invasion. However, many aspects of the locust cellular defense system are not well understood, which includes the process of hemocyte recruitment and replenishment. The hemocytes may be sacrificed during infection due to terminal events such as lyses to release pathogen toxic compounds into the hemolymph, or due to nodule/capsule formations, or simply by hemocyte coagulation during injury and wound healing (Jiravanichpaisal et al., 2006; Lavine and Strand, 2002; Oliver et al., 2011). The cellular defense may then demand a high level of hemocyte proliferation to replenish the sacrificed hemocytes. However, the pattern of hemocyte proliferation in locusts, which is an underlying component of the cellular defense, is still poorly investigated. The existing knowledge is based on an old hypothesis which assumes that hemocyte losses in locusts during injury/infection may be caused by a temporary functional switch in the hematopoietic tissue (Grigorian and Hartenstein, 2013; Hoffmann et al., 1974; Hoffmann, 1972). Following infection, the locust hematopoietic tissue is assumed to temporarily shut down the hemocyte production process as it dedicates itself to eliminate the pathogen, leading to a reduction in a total count of the circulating hemocytes. This hypothesis clearly undermines the hemocytes' role in the locust cellular defense and may direct readers to misleading conclusion about the locust hematopoietic tissue.

Therefore, we did extensive immunofluorescence-based assays to investigate the locust cellular immune system, especially by addressing the pattern of hemocyte proliferation during an infection. We studied immediate effects of an infection on the hemocytes and hematopoietic tissue by doing total hemocyte count and apoptotic tests. We investigated the pattern of hemocyte proliferation following infections by using discriminating markers for DNA synthesis and mitosis. We report herein that, following infection, the circulating hemocytes undergo active proliferation to replenish themselves, and that they, not the hematopoietic tissue, are the primary sites of hemocyte production in adult locusts.

2. Materials and methods

2.1. Locust stocks

We used the locust *Locusta migratoria* for our experiment. The locusts were reared in ventilated cages (1 m × 0.5 m × 0.5 m) at high density (200–300 locusts per cage), representing crowded conditions. They were fed daily with fresh grass and dried oat flakes. The environment in the locust room was regulated so that the temperature, photoperiod and relative humidity were 32 ± 1 °C, 13 h of light and 40–60%, respectively. Eggs from mature female locusts were deposited in pots containing moist and sterile sand (sand:peat:water ratio of 7:3:1). The pots were then transferred to new cages every week so that each cage contains a pool of first instars with age difference less than 7 days. The locusts were synchronized immediately after the final molt and monitored in new cages. Adult locusts used for the experiment aged between 1 and 5 days after final molt. When instars were used, they were collected from a single cage per experiment. Each experiment was done with at least five locust individuals per condition and was repeated three times.

2.2. Injection and total hemocyte count

Experimental injections were made with Hamilton-microsyringe (10 µL final volume) by penetrating between the second and the third ventral abdominal disks. The fungal membrane component β-1,3-glucan (Sigma-Aldrich) was injected as an immunogen at a dose of 100 µg per insect, as previously described (Gunnarsson, 1988; Wang et al., 2013). Locusts were anesthetized with carbon dioxide prior to injections and hemolymph collections.

Total hemocyte count in adult locusts was determined according to established procedures (Hoffmann et al., 1974; Söderhäll et al., 2003). Prior to injection, hemolymph was collected from all locusts and hemocyte density (cells per µL hemolymph) in normal circulation was estimated. The locusts were then injected with β-1,3-glucan in NaCl/Pi buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2) or with NaCl/Pi (control), and each locust was followed for change in hemocyte density by counting hemocytes at different times (30 min to 72 h) post injection. At each time point, 10 µL of hemolymph was collected and immediately mixed with 90 µL of ice-cold anti-coagulant solution (98 mM NaOH, 186 mM NaCl, 17 mM Na_2EDTA, 41 mM citric acid, pH 4.5). Hemocyte counts were then made by a cell counter (NucleoCounter® NC-100™) and hemocyte density was calculated.

2.3. Hemocyte harvesting and fixation

Hemocytes were harvested for characterization from each adult locust as follows. First, locusts were injected with 300 µL anti-coagulant solution. Within 1 min after injection, the base of the hind-leg was pierced by a fine needle and the hemolymph was flushed out on a coated glass slide (Superfrost®Plus). Next, it was quickly spread on the slide and dried on a prewarmed slide drying bench. The slide was briefly washed with NaCl/Pi and fixed in 4% paraformaldehyde (Sigma-Aldrich) in NaCl/Pi 1 h at room temperature (RT). Finally, the slide was washed 3× with NaCl/Pi buffer and stored in NaCl/Pi containing 0.1% NaN_3 at 4 °C until use.

2.4. Hematopoietic tissue preparation

The locusts were killed by decapitation and the abdominal regions containing the HPT were cut off from the rest of the body using fine scissors (Fig. 1B). Next, the HPT was prepared for examination as follows, unless described otherwise. The tissue was washed with NaCl/Pi to remove residual circulating hemocytes and fixed in 4% paraformaldehyde at RT overnight. After rinsing in NaCl/Pi and distilled water, the HPT was dehydrated in an ethanol-xylol series. Afterwards, it was embedded in paraffin and cut into 7 µm transverse sections. The sections were attached on the coated glass slides and kept at RT until use. A representative slide for each tissue section was prepared and stained with hematoxylin and eosin (H&E). The tissue sections were deparaffinized in xylene and rehydrated in ethanol (100–50%) and distilled water prior to H&E and immunofluorescent stainings.

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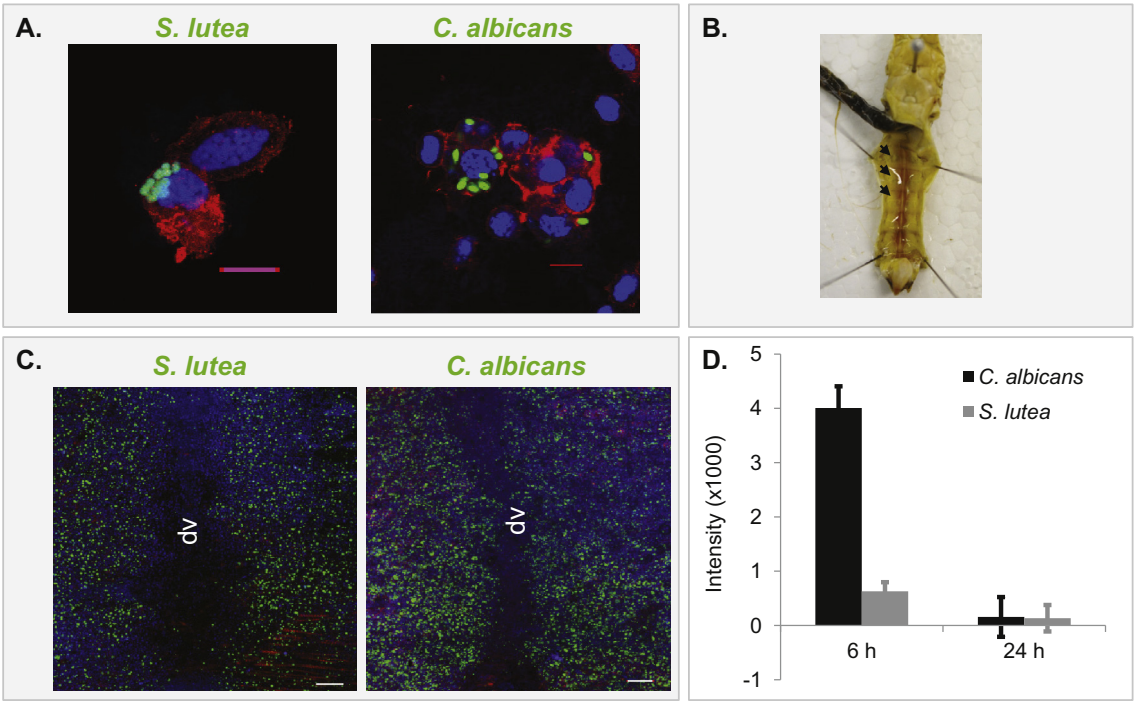

Fig. 1. Pathogen clearance by locust hemocytes and hematopoietic tissue. (A) Phagocytotic hemocytes identified in the circulation 6 h post injection of *S. lutea* (left, green) and *C. albicans* (right, green). Scale bars 10 μm. (B) The adult locust hematopoietic tissue (black arrows indicate the abdominal pericardial region where the hematopoietic tissue resides). (C) The hematopoietic tissues after the locusts were challenged for 6 h with *S. lutea* (left, green) and *C. albicans* (right, green). The largest proportions of the injected microbes were cleared from the circulation by the hematopoietic tissue. Scale bars 1 mm. DAPI (blue), Phalloidin (red), dv (dorsal vessel). (D) Extracellular fluorescent intensity determined in the locust hemolymph after injections of FITC-labeled *S. lutea* and *C. albicans*. Zero intensity indicates total bacterial/fungal clearance from the circulation ($P_{6h} < 0.005$, $P_{24h} > 0.300$). Error bars designate standard error of the mean (SEM).

2.5. Phagocytosis assay

S. lutea and *C. albicans* were fluorescently labeled as described in supplementary methods S1 (Wynant et al., 2014) *and* adult locusts were injected with 10 μL of the FITC-labeled *S. lutea* or *C. albicans*. After 6 h, the locusts were injected again with 200 μL trypan blue solution (0.4% in NaCl/Pi) and incubated for 15 min to quench any extracellular fluorescence. The hemocytes and HPT were then harvested as described in Sections 2.3 and 2.4, and fixed after removing excess trypan blue by brief washing with NaCl/Pi. The HPT was washed 15 min with 3% TritonX-100 in TBS for whole-mount staining. Both the hemocytes and the HPT were then stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and Alexa-Fluor® 546 phalloidin (Life Technologies) for 1 h, and later mounted with Mowiol® 4-88 (Sigma-Aldrich) for microscopy.

2.6. BrdU treatment techniques

As in previous studies (Illa-Bochaca and Montuenga, 2006; Söderhäll et al., 2003; Wojtowicz and Kee, 2006), *in vivo* DNA synthesis in the locusts was determined by 5-bromo-2'-deoxyuridine (BrdU) labeling. The locusts were injected once with 100 μg BrdU (Sigma-Aldrich) for tests up to 30 h. For studies between 2 and 11 days, repeated BrdU injections (24 h, 3 days and 5 days post the initial administration) were made to ensure BrdU labeling over the extended period.

2.7. BrdU detection and mitosis studies

Antibodies were used to detect BrdU labeled cells and histone H3 Serine 10 phosphorylation (H3S10P) in mitotic cells. The hemocytes and HPT slides prepared as stated in Sections 2.3 and 2.4 were placed in prewarmed 10 mM citrate buffer (10 mM trisodium citrate dihydrate, pH 6.0) containing Triton X-100 (0.05%) and incubated in a microwave (10 min at 750 W and 5 min at 600 W). After 20 min cooling, they were permeabilized by washing with 3% TritonX-100 in TBS (15 min), and incubated at RT 1 h in blocking buffer, overnight in primary antibodies and 2 h in secondary antibodies. The primary antibodies used were 1:300 rat anti-BrdU (ABD Serotec) or 1:500 rabbit anti-H3S10P (Millipore). The secondary antibodies were 1:200 Alexa-Fluor® 594 anti-rat or 1:200 Alexa-Fluor® 488 anti-rabbit (Life Technologies). The slides were washed with 3% TritonX-100 in TBS for 15 min between each step. For negative control, only secondary antibodies were used for staining. The slides were finally counterstained with DAPI for 30 min and mounted with Mowiol® 4-88 for microscopy.

2.8. Cell death detection

In situ cell death assays in HPT was done by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection method (Roche), according to the manufacturer's instructions. In short, a rehydrated tissue section was placed in 10 mM citrate buffer containing Triton X-100 (0.05%), heated in a microwave (750 W) for 1 min and quickly cooled by adding 80 mL distilled water. After permeabilization with 3% TritonX-100 in TBS (15 min), the tissue was incubated in blocking reagent (10 mM Tris-HCl, pH 7.5, containing 3% BSA and 20% FBS) at RT for 1 h. Next, it was rinsed in PBS, incubated in TUNEL reaction mixture in the dark at 37 °C in a humid atmosphere for 1 h, rinsed again in PBS and counterstained with DAPI. Controls were included according to the manufacturer's guide.

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2.9. Microscopy and data analysis

Photos were taken with Zeiss microscope (Imager.Z1) and processed with ZEN 2012 (blue edition). ImageJ software was used for image data quantification. Percentage of BrdU labeled cells were calculated by dividing the total number of BrdU cells by the total number of cells at a particular time point. Mitotic index was determined in a similar way (mitotic cells divided by total cells). All statistical inferences were made based on ANOVA and Fisher's *t*-test.

3. Results

3.1. Pathogen clearance by locust hemocytes and hematopoietic tissue

Adult locusts were injected with heat-deactivated and FITC-labeled gram positive bacterium *S. lutea* and the yeast *C. albicans*. After 6 h of incubation, extracellular fluorescence in the hemolymph was quenched by trypan blue treatment and, the hemocytes and HPT were collected for observation. The HPT engulfed an enormous amount of the injected microbes (Fig. 1C). This was expected as the HPT in locusts is strongly phagocytotic and removes continuously cell debris and foreign particles from the hemolymph (Hoffmann et al., 1979). Several of the hemocytes also endocytosed the injected microbes, and multiple microbes were cleared at once by single circulating hemocytes (Fig. 1A). Whereas the circulating hemocytes removed a small part of the microbes injected in the hemolymph, the mutual action of the HPT and hemocytes totally cleared the injected microbes within 24 h post challenge (Fig. 1D).

3.2. Primary response of adult locusts to infection includes release of newly synthesized hemocytes into the circulation

Previous studies showed that, in adult locusts, hemogram changes, such as circulating hemocyte depletion, take place due to injury or infection although they poorly addressed the process of hemocyte replenishment, which is vital for a sustained cellular defense (Gunnarsson, 1988; Hoffmann et al., 1974; Hoffmann, 1972). We did revalidation tests by treating adult locusts with a fungal membrane component β-1,3-glucan. The locusts lost over 60% of the circulating hemocytes within 30 min following β-1,3-glucan injection (Fig. 2A). Besides, the density of the circulating hemocytes remained at lowest level for over 3 h. The process of hemocyte replenishment, a rise in the number of the circulating hemocytes following the depletion, seemed to start in 6 h after the immune challenge, at which point over 10% of the lost hemocytes were replenished. A full recovery of hemocyte count required at least 48 h when the total hemocyte density in immune-challenged locusts reached on average 87% of the pre-injected level.

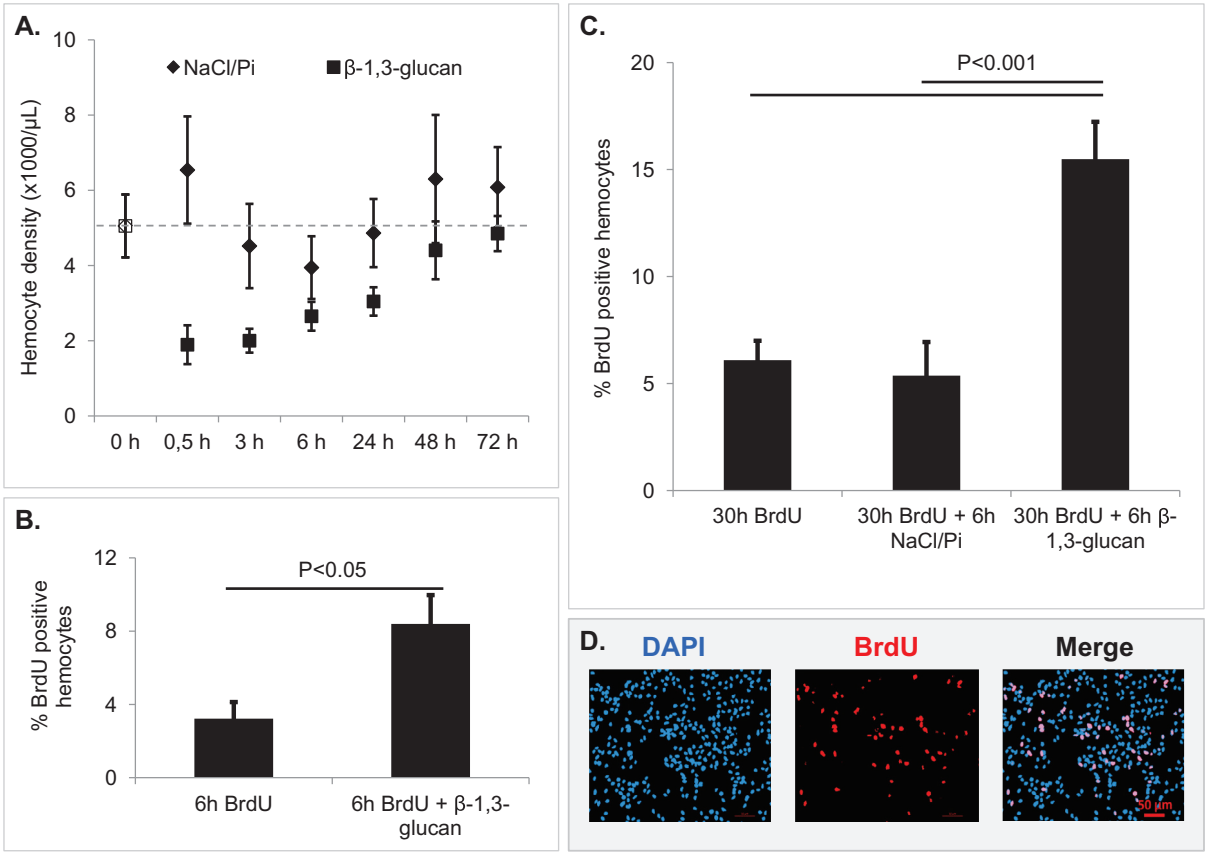

Fig. 2. Hemocyte depletion and replenishment after β-1,3-glucan injection in adult locusts. (A) Total hemocyte density following injections of β-1,3-glucan and control NaCl/Pi buffer (two-way ANOVA *P*-values <0.001). Each data point is presented as mean ± 95% confidence interval of the mean. The β-1,3-glucan injection induced hemocyte depletion as the hemocyte counts were significantly lowest at 0.5 and 3 h post injection ($P < 0.0001$). The hemocyte count returned to a normal level at 48 h ($P = 0.267$) post injection. (B) Comparison of BrdU incorporation by the circulating hemocytes between locusts administered with BrdU for 6 h in the absence and presence of β-1,3-glucan. The number of BrdU labeled hemocytes in the circulation increased more than two-folds in the presence of β-1,3-glucan (error bars show SEM). (C) Comparison of BrdU labeling in the circulating hemocytes between locusts administered with BrdU for 30 h and locusts challenged with β-1,3-glucan for 6 h after 24 h BrdU administration. The β-1,3-glucan challenge significantly increased the number of BrdU labeled hemocytes in the circulation (error bars show SEM). (D) Fluorescent image of circulating hemocytes in 30 h BrdU labeled and 6 h β-1,3-glucan challenged locusts.

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We studied hemocyte production in adult locusts following the hemocyte loss by using BrdU, a marker for new DNA synthesis. In naïve locusts, about 3% of the circulating hemocytes became BrdU-labeled when treated for 6 h (Fig. 2B). Simultaneous injection of BrdU and β-1,3-glucan increased the proportion more than twofolds (8.4%), indicating that hemocyte precursor cells might be induced to undergo proliferation. To validate this assumption, locusts were first administered with BrdU and incubated for 24 h. Afterwards, they were challenged with β-1,3-glucan and BrdU-labeled hemocytes were determined 6 h post challenge (Fig. 2C and D). As anticipated, the proportion of the BrdU-labeled cells in the circulating hemocytes of the immunized locusts significantly increased to over 15% while only 6% of the hemocytes in the control locusts incorporated BrdU. The data showed that in the locusts hemocyte depletion following infection induces the proliferation of hemocyte precursor cells to replenish the lost cells in the hemocoel.

The BrdU clearance in adult locusts was estimated by labeling Bm5 cells *in vitro* with hemolymph plasma collected at different time points post BrdU injection. BrdU resides in the locust hemolymph at least for 6 h and is totally cleared from the hemolymph at 24 h post injection. The data altogether implied that in adult locusts only a small cell population may be taking part in cell proliferation as a set of hemocyte precursor cells. An immune challenge with β-1,3-glucan seems to affect this same population by making them more actively dividing.

3.3. Infection induces substantial cell death but no cell division in the hematopoietic tissue of adult locusts

An examination of hematoxylin and eosin (H&E) stains showed that high cell density in the locust HPT is mainly present in the first two dorsal abdominal disks (anterior regions). The cell accumulation in the posterior region of the HPT declines progressively to none. Hence, we present in this manuscript our data collected for the anterior region. We would also like to mention that we did not observe any difference between the anterior and the posterior region to our treatments.

Injection of β-1,3-glucan causes loss of cells in the HPT but no cell proliferation. After 6 h of β-1,3-glucan treatment, the locust HPT was collected, transversely sectioned and processed for TUNEL staining. The HPT tissue is normally TUNEL negative in naïve locusts. However, the β-1,3-glucan injection causes nodule formation all over the tissue and turns a significant section of the tissue into TUNEL positive, indicating an induction of substantial cell death in the tissue (Fig. 3A and B). The apoptotic cells were observed until 24 h post β-1,3-glucan treatment (Fig. S1).

We investigated the effect of infection on cell proliferation in the HPT. To our surprise, BrdU-labeled cells were not detected in the HPT and infection did not change the scenario during the 30 h studies (Fig. 4A and B). In addition, dual stains for BrdU-labeling and mitotic activity showed a rare presence of mitotic cells in the tissue during

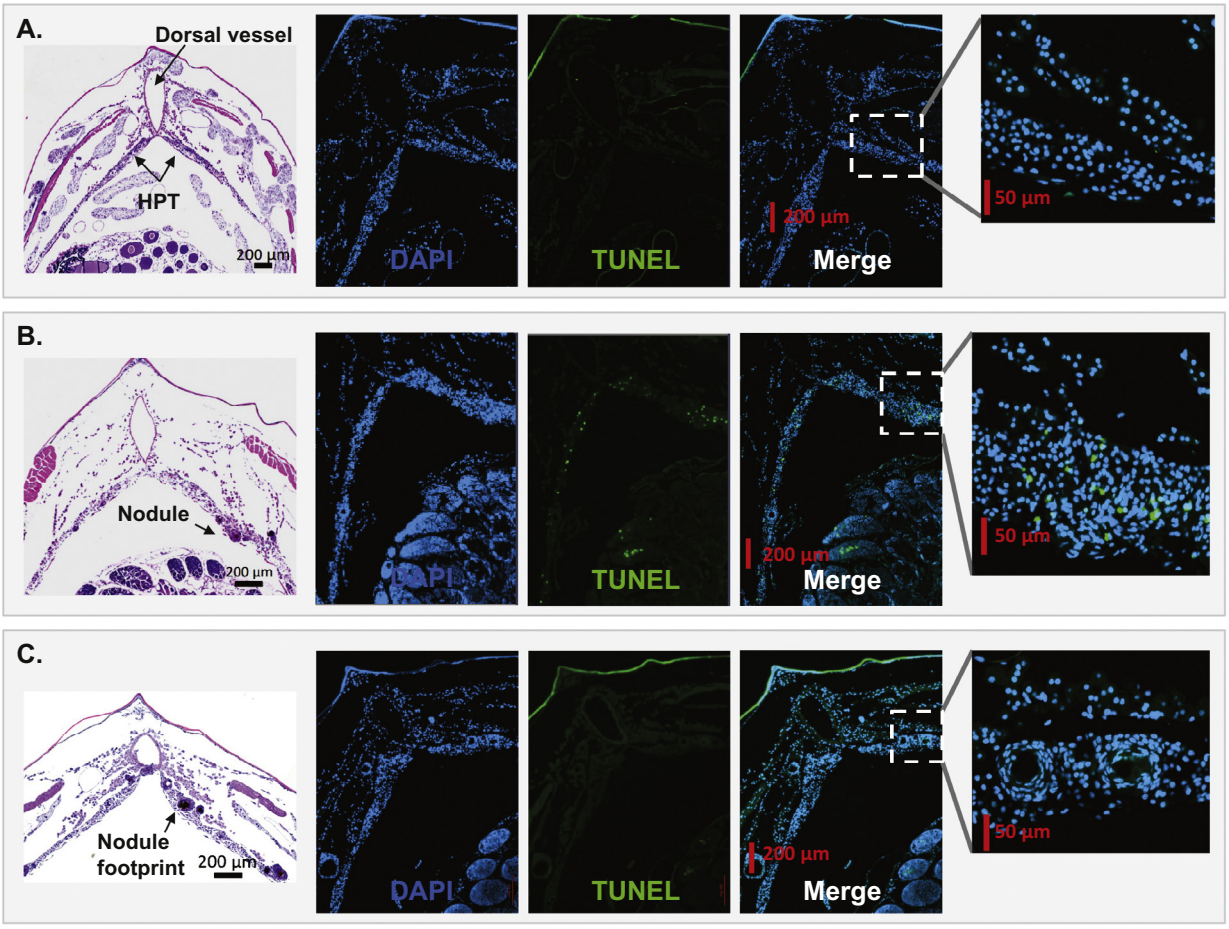

Fig. 3. Infection induces massive cell death in hematopoietic tissue of adult locusts. Hematoxylin eosin (left) and TUNEL fluorescent (right) staining of the HPT in naive locusts (A), in locusts 6 h (B) and 3 days (C) post β-1,3-glucan challenge. The HPT is normally TUNEL negative (no apoptotic cells). The β-1,3-glucan challenge induces nodule formation and massive cell death (TUNEL positive) in the tissue. The TUNEL positive cells disappear after 3 days.

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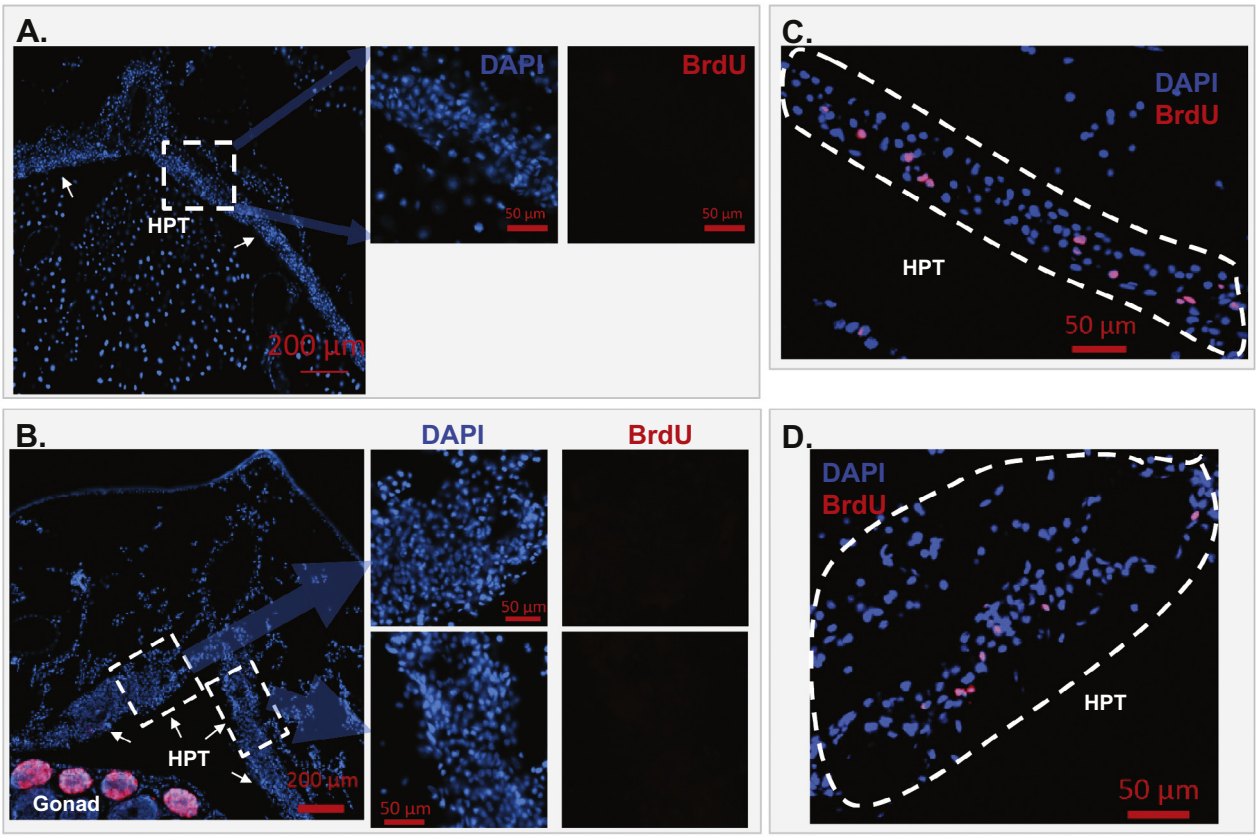

Fig. 4. Cell proliferation in hematopoietic tissue of adult locusts. BrdU incorporation by the HPT (A) after 30 h BrdU administration and (B) after 30 h BrdU administration with 6 h post β-1,3-glucan challenge. The HPT did not incorporate BrdU up to 24 h post treatment and β-1,3-glucan injection did not change the status quo. BrdU incorporation by the HPT (C) after 48 h BrdU administration and (D) after 48 h BrdU administration with 24 h post β-1,3-glucan challenge. In 48 h treatments, a similar number of BrdU labeled cells started to be detected both in naïve and immune challenged individuals.

the 30 h studies (Fig. 5A). The mitotic cells identified were BrdU-negative and do not seem to be induced by the β-1,3-glucan challenge. The DNA duplication stage prior to mitosis in the cell cycle of the dividing cells seemed to have taken place before the onset of the 30 h studies, suggesting also no association between the mitotic cells and our experiment. Hence, the lack of actively dividing cells implied that the HPT may not be a primary source for hemocyte production in the adult locusts.

BrdU-labeled cells in the HPT started to be detected in a similar fashion since 48 h of BrdU administration both in naïve and immune challenged individuals (Fig. 4C and D). Regardless, given that the circulating hemocytes are already BrdU-labeled by this time, it was not possible to confirm whether or not some of the labeled HPT cells might be discharged into the circulation at this time point.

3.4. *Circulating hemocytes are the primary sources for new hemocyte production in adult locusts*

Previous studies speculated that the hematopoietic tissue in the locusts is a primary source of new hemocyte production (Grigorian and Hartenstein, 2013; Hoffmann et al., 1974; Hoffmann, 1972). The observed absence of actively proliferating cells in the HPT demanded further investigation of the circulating hemocytes. As expected, we identified a small fraction of the circulating hemocytes (0.25%) being mitotic (Fig. 5B and C). Most of the mitotic cells were also actively dividing as they were double labeled for BrdU and H3S10P within 24 h post BrdU administration. A β-1,3-glucan challenge significantly increased the number of mitotic cells in the circulation (1.6%) in 3 h. The intensified cell division was also observed up to 6 h post challenge (Fig. 5B). This is in agreement with our BrdU-labeling data (Fig. 2) by which we demonstrated an induction of hemocyte production in the locusts following an infection. Therefore, the overall data show that the circulating hemocytes in the adult locusts can replenish themselves during infection and are indeed the primary sources of new hemocyte production.

3.5. *The hematopoietic tissue grows continuously in cell number and tissue size despite infection*

Given that the locust HPT plays a primary role in the pathogen clearance, the dying cells in the tissue in the course of infection should be continuously replenished to maintain the cellular defense. The HPT shows constant growth in total cell mass since early larval stages (Fig. S2) besides a very low rate of cell death in the naïve individuals (Fig. 3A), thereby creating a high accumulation of potential phagocytotic cells in adults. The cell proliferation studies showed a considerable number of BrdU-labeled cells in the adult HPT after 4 days of BrdU administration (Fig. 6A, B, E). The proportion of BrdU-labeled cells reached maximum after 6 days when a substantial section of the HPT became BrdU positive (Fig. 6C–E). Regardless, the β-1,3-glucan challenge did not affect this cycle of the HPT regeneration (Fig. 6E). Dual TUNEL and BrdU tests showed that the apoptotic cells induced in the HPT following the infection were cleared within 3 days with no change in the activities of the tissue cell proliferation, thereby suggesting a restoration of the tissue to normal physiology (Figs. 3C, 6A and B). In addition, investigation of the adult HPT for extended periods (up to 11 days) showed that the

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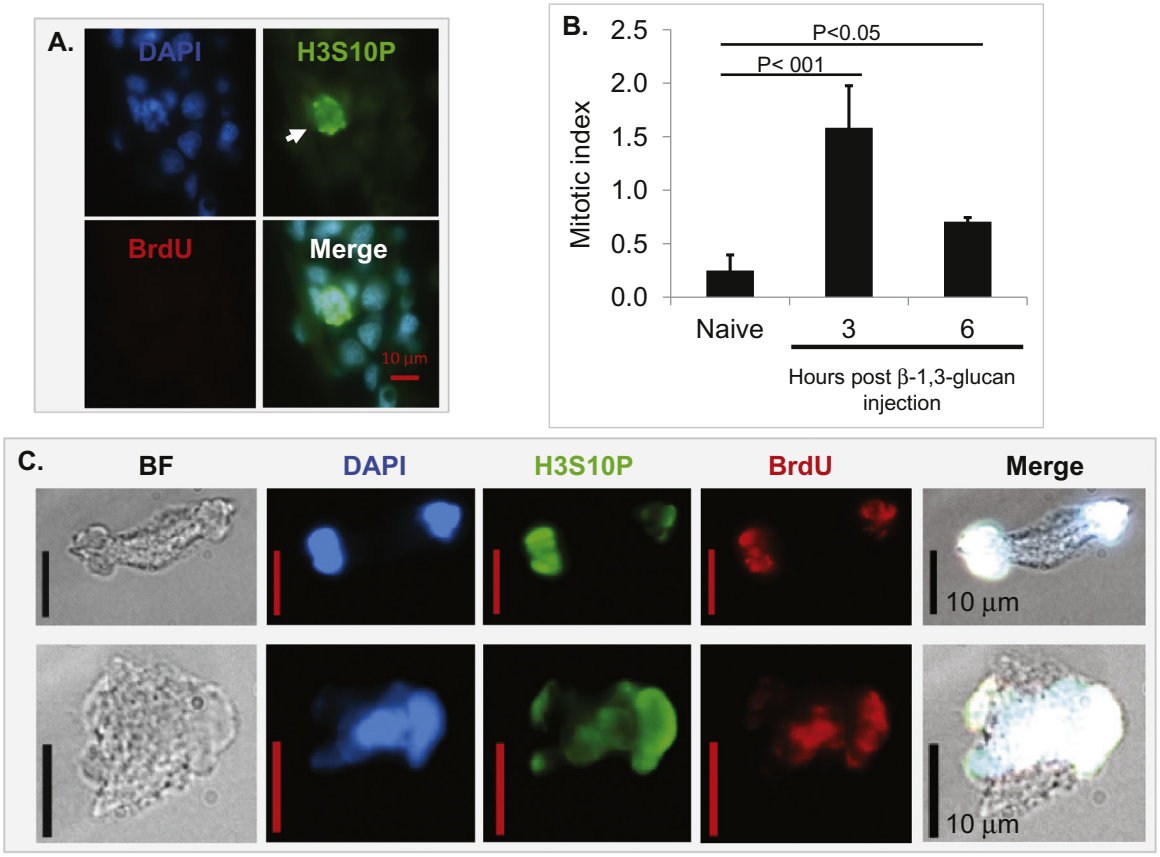

Fig. 5. Mitotic activity by hematopoietic tissue and circulating hemocytes in adult locusts. (A) Dual immunofluorescent image of the HPT 30 h post BrdU administration. Despite infection, detected mitotic cells (H3S10P labeled) in the HPT during this time period did not incorporate BrdU. (B) Mitotic index of the circulating hemocytes in naïve and β-1,3-glucan challenged locusts. A small portion of the hemocytes in the naïve locusts undergoes proliferation and their number significantly increases post β-1,3-glucan challenge. Error bars designate SEM. (C) Bright field and dual fluorescent images of the circulating hemocytes that are undergoing active proliferation (both BrdU and H3S10P labeled cells).

production of new cells continues in the peripheral regions of the tissue while infection footprints such as nodule sites remain vacant (Fig. 7). Therefore, the persevering response of the locust HPT during infection may eventually be explained by its basic nature, which includes a modest regeneration period and high accumulation of phagocytes.

4. Discussion

Insects lack adaptive immunity, which means, in times when the cuticle is breached, they must always mobilize their innate cellular and humoral machineries to defend themselves against pathogens (Beutler, 2004; Hultmark, 1993; Siva-Jothy et al., 2005). The humoral immune system has been deeply studied in many insect species, including the locusts, and many insect humoral factors that have vertebrate homologs are identified (Imler, 2014; Lavine and Strand, 2002). For some reasons, the cellular defense in insects, except for some dipteran and lepidopteran species, has obtained little attention in the past decades (Beutler, 2004; Fauvarque and Williams, 2011; Hultmark, 1993; Imler, 2014; King and Hillyer, 2013; Tan et al., 2013). The role of hemocytes, which includes pathogen clearance and hemocyte proliferation during the cellular response, is especially poorly elaborated (King and Hillyer, 2013; Oliver et al., 2011; Tan et al., 2013). Hence, we present herein data on the locust cellular defense, as the Orthopteran model, especially by addressing the role of the circulating hemocytes and the HPT in the locust cellular defense system.

The hemocytes and HPT mutually clear pathogen particles from the hemocoel. Our data show the HPT as the remover of the largest portion of bacterial and fungal pathogens in the hemolymph. This result was obviously expected since the HPT is known as the principal tissue that maintains the sanitation of the hemolymph by removing cell debris and any foreign particles (Hoffmann et al., 1979). The circulating hemocytes eliminate also a handful of the pathogens from the circulation via phagocytosis of multiple pathogenic cells per hemocyte. Most importantly, our observation about induced loss of circulating hemocytes following infection in the locusts was consistent with earlier studies (Gunnarsson, 1988; Hoffmann et al., 1974; Hoffmann, 1972). Our research additionally addressed the process of hemocyte replenishment as the sudden and massive drop in hemocyte population may not be beneficial for the locust, especially in the continuity of the host defense. In particular, we wanted to identify the principal sources for the hemocyte replenishment.

As early as 6 h post immune challenge, the locusts responded to the hemocyte loss by releasing new hemocytes into the circulation. The released hemocytes were identified mainly as newly synthesized hemocytes, as evidenced by the BrdU-based cell proliferation test. A dual detection of BrdU incorporation and mitotic activity further confirms actively dividing cells and allows verification of the main source of the hemocyte production (King and Hillyer, 2013; Noonin et al., 2012; Tan et al., 2013). Whereas a small portion of the naïve circulating hemocyte population contains actively dividing cells, the proportion of the mitotic cells rises immediately following an infection (3 h). In contrast, the HPT does

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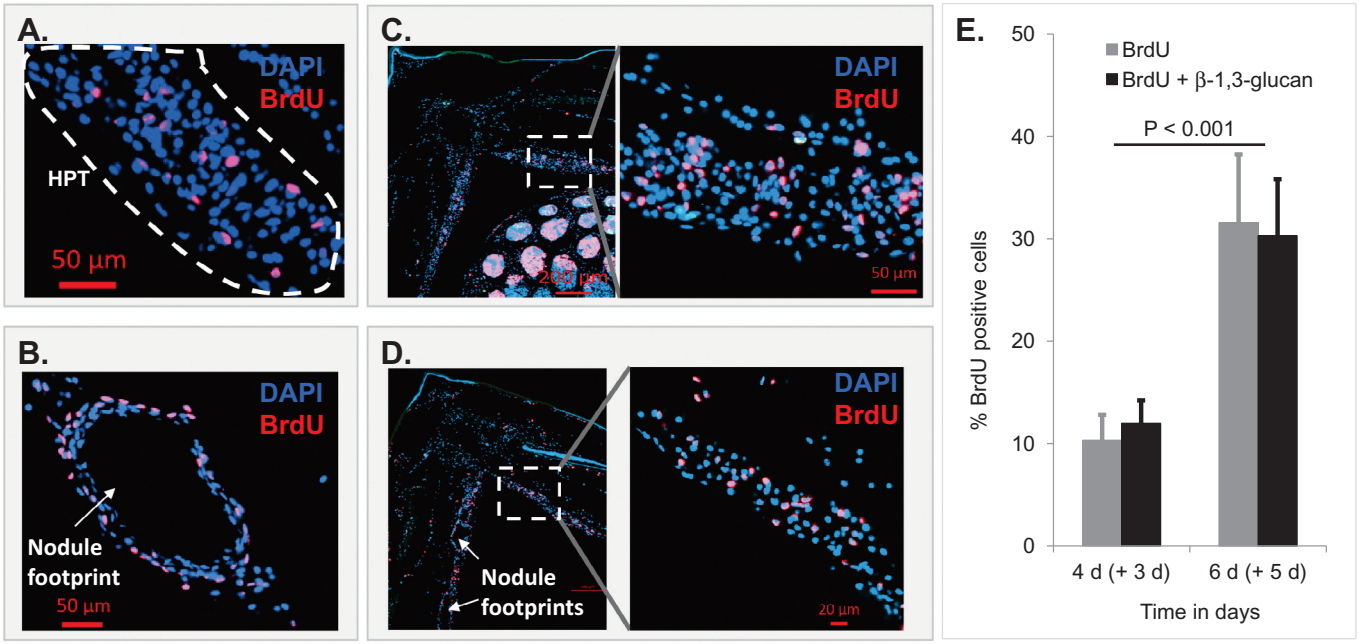

Fig. 6. Long term effects of infection on proliferation in hematopoietic tissues of adult locusts. BrdU incorporation by the HPT (A) after 4 days of BrdU administration and (B) after 4 days of BrdU labeling with 3 days post β-1,3-glucan challenge. During this time period, a few number of BrdU positive cells were detected in the tissue despite infection. The HPT (C) after 6 days of BrdU administration and (D) after 6 days of BrdU administration with 5 days post β-1,3-glucan challenge. At this time point, a substantial section of the tissue became BrdU positive both in the naïve and β-1,3-glucan treated individuals. (E) Quantification of data presented in A–D (two-way ANOVA $P_{days} < 0.001$, $P_{treatment} = 0.988$, $P_{days*treatment} = 0.583$). Days in parentheses indicate days post β-1,3-glucan injection. Error bars show SEM.

not incorporate BrdU for extended periods (30 h) and rarely contains dividing cells, suggesting no link between the tissue and the new hemocytes. The HPT in adult locusts, however, has long been thought to be a primary source of hemocyte production although no active cell differentiation was detected in the tissue (Grigorian and Hartenstein, 2013; Hoffmann, 1972, 1973; Hoffmann et al., 1979). This assumption was mainly based on indirect evidence as an HPT destruction by X-irradiation led to hemocyte depletion, particularly coagulocytes and granulocytes, followed by 5 days of hemocyte and HPT recovery. Such assumption seems to undermine the circulating hemocytes since a tissue lesion by itself might attribute to a significant loss of the hemocytes due to subsequent clotting reactions (Jiravanichpaisal et al., 2006; Lavine and Strand, 2002; Siva-Jothy et al., 2005; Theopold et al., 2004). Additionally, it has been advised in general that cell lineages are identified best with direct visualization experiments (Morrison and Spradling, 2008; Orkin and Zon, 2008). Therefore, we report here that the primary sources for hemocyte replenishment following infection in adult locusts are actually the circulating hemocytes. Our observations regarding hemocyte production in adult stages of the hemimetabolous

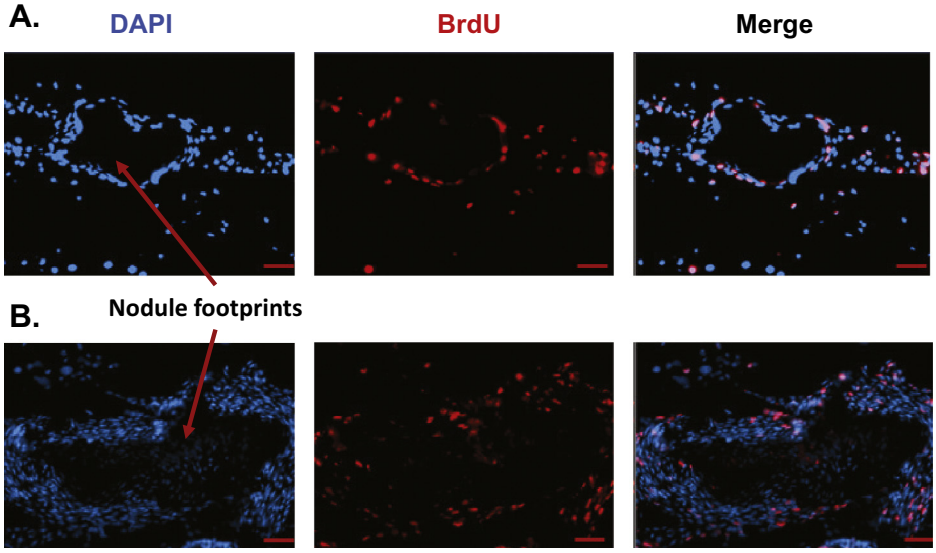

Fig. 7. Immunofluorescent staining for determining the fate of nodules in the adult hematopoietic tissue. Nodule footprints in the HPT (A) 5 days post β-1,3-glucan challenge with 6 days BrdU administration and (B) 10 days post β-1,3-glucan challenge with 11 days BrdU administration. The nodule footprints remain unfilled while new cells were continuously supplied to the nodule peripheral regions. Scale bars 50 µm.

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locust, in the end, also seem to be not different from the situation described in holometabolous insects such as *Drosophila* (Crozatier and Meister, 2007), mosquito (King and Hillyer, 2013) and silkworm (Tan et al., 2013).

The cell density in the insect HPT changes during growth as the HPT undergoes a massive proliferation/differentiation at the onset of each molt (Grigorian and Hartenstein, 2013; Hoffmann et al., 1979; Jung et al., 2005; Lanot et al., 2001). This is associated with the role the HPT plays during the molting process, for instance in removing cell debris from the hemocoel (Crozatier and Meister, 2007; Hoffmann et al., 1979; Lanot et al., 2001). After the final molt, the HPT degenerates in the adult stage of many insects while it seems to continuously prevail in the locusts (Grigorian and Hartenstein, 2013; Lanot et al., 2001; Nardi et al., 2003). Our data, as in previous studies, show that the adult HPT in locusts requires a modest period (4–6 days) for regeneration (Hoffmann et al., 1974; Hoffmann, 1972) and the turnover rate seems to ensure a continued cell supply to the tissue. As the locust reaches the flight stage (5–10 days after the final molt), the tissue accumulates already an enormous amount of cells, which are potentially phagocytotic (Figs. 1C and 6A–D). This seems to boost the locust cellular defense given that the HPT resides in the abdominal pericardial region where the hemolymph has to flow before directed to the open cavity (Chapman et al., 2013). Therefore, the pathogens after penetrating the cuticle may most likely come in contact with the HPT before reaching to other parts. This way, the HPT, with its abundant phagocytes, may ensure a continuous removal of the pathogens from the cavity to safeguard the host. The HPT may also ease the burden on the circulating hemocytes to clear the pathogens, which may explain why the hemocytes take extended periods (over 24 h) to fully replenish the depleted cells in the circulation following an infection.

In adult locusts, it has been hypothesized that the HPT undergoes a functional switch from an actively dividing tissue to a quiescent but a highly phagocytotic tissue immediately after infection (Hoffmann et al., 1974, 1979). The HPT proliferation was assumed to resume at intensified rate at later stages (2–5 days post infection). To our surprise, we did not see active proliferation in the tissue regardless of infection. Besides, infection did not change the status quo of the HPT proliferation (up to 11 days). Our data suggest that the old hypothesis is incorrect and that the HPT rather shows a persevering response to infection as we described earlier. Despite infection, the adult HPT seems to continually accumulate phagocytotic cells by undergoing tissue regeneration (4–6 days). This process seems to offer a prophylactic advantage to the locusts; thereby arming them with an enormous number of defense cells prior to an infection and avoiding an induction of massive cell production following the infection. Previous studies show that the locusts during crowding and flight stages actually boost their prophylactic immune system to avoid energy loss due to pathogen offenses (Siva-Jothy et al., 2005; Wang et al., 2013; Wilson et al., 2002). Given the combined physiological advantages (anatomical, cytological and immunological) the HPT offers, we hypothesize that the tissue may be used as one prophylactic tool for the locust host defense. The HPT might safeguard the locust by containing pathogens, especially during long distance flights when the locust needs energy the most for flight and at the same time is constantly exposed to a wide variety of pathogens.

Despite our research outcomes, there are still some other hypotheses which require further investigation by making use of tissue specific markers. One is an assumption of immediate release of premature hematopoietic cells (reticular cells) into the hemolymph following excessive bleeding in locust instars (Hoffmann, 1969). In the adult HPT, given the absence of related BrdU labeled parental cells to the supposedly "premature reticular cells" released into the circulation, our data could not support this hypothesis. The other is the probability that the HPT in adult locusts might play a secondary role in hemocyte production. Despite delayed BrdU-labeling of the HPT and lack of induced proliferation following infection in this tissue, this possibility could not still be ruled out with our data since both the HPT and circulating hemocytes became BrdU labeled during the late phases of our studies.

In conclusion, our data show new insights in the locust cellular defense. The circulating hemocytes in adult locusts are the primary sources of new hemocyte production while the HPT exhibits rather phagocytotic than hematopoietic nature. While both the circulating hemocytes and the HPT participate in the cellular host defense, the HPT takes the lion share in clearing pathogens from the circulation. In addition, as the HPT prevails in adult stages and remains strongly phagocytotic, it may be one of the prophylactic tools that the locust developed to utilize during long distance flights.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.dci.2014.09.005.

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